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Altered Trafficking of Membrane Proteins in Purkinje Cells of *SCA1* Transgenic Mice

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Spinocerebellar ataxia type 1 (SCA1) is a neurodegenerative disease caused by the expression of mutant ataxin-1 that contains an expanded polyglutamine tract. Overexpression of mutant ataxin-1 in Purkinje cells of transgenic mice results in a progressive ataxia and Purkinje cell pathology that are very similar to those seen in SCA1 patients. Two prominent aspects of pathology in the *SCA1* mice are the presence of cytoplasmic vacuoles and dendritic atrophy. We found that the vacuoles in Purkinje cells seem to originate as large invaginations of the outer cell membrane. The cytoplasmic vacuoles contained proteins from the somatodendritic membrane, including mGluR1, GluRΔ1/Δ2, GluR2/3, and protein kinase C (PKC) γ. Further examination of PKCγ revealed that its sequestration into cytoplasmic vacuoles was accompanied by concurrent loss of PKCγ localization at the Purkinje cell dendritic membrane and decreased detection of PKCγ by Western blot analysis. In addition, the vacuoles were immunoreactive for components of the ubiquitin/proteasome degradative pathway. These findings present a link between vacuole formation and loss of dendrites in Purkinje cells of *SCA1* mice and indicate that altered somatodendritic membrane trafficking and loss of proteins including PKCγ, are a part of the neuronal dysfunction in *SCA1* transgenic mice. *(Am J Pathol 2001, 159:905–913)*

Spinocerebellar ataxia type 1 (SCA1) is an autosomal-dominant, progressive neurodegenerative disease. In SCA1, the primary cellular sites of neurodegeneration are Purkinje cells of the cerebellar cortex and a select population of neurons in the brainstem. These neurodegenerative changes lead to the characteristic ataxia and bulbar dysfunction seen in SCA1 patients. The disease is caused by the expansion of an unstable CAG repeat within the *SCA1* gene.[1] Because this trinucleotide repeat is located within the coding region of *SCA1*, the expansion results in a longer polyglutamine tract within the protein designated ataxin-1. SCA1 is a member of a class of neurological disorders, that includes Huntington disease, spinobulbar muscular atrophy, dentatorubropallidoluysian atrophy, and the spinocerebellar ataxias (SCA1, SCA2, SCA3/MJD, SCA6, and SCA7).[2] Each is caused by the expression of a mutant polyglutamine protein.

By overexpressing a full-length *SCA1* cDNA-encoding mutant ataxin-1 with 82 glutamines under the direction of the Purkinje cell-specific *Pcp2/L7* promoter, we established transgenic mice that develop a progressive ataxia.[3,4] These *SCA1* transgenic mice have provided several important insights into the molecular basis of this polyglutamine-induced disease. In these mice, before the onset of ataxia, multiple pathological alterations were detected in Purkinje cells. At 3 weeks of age, large vacuoles were detected in the cell bodies of many Purkinje cells.[3,4] Electron micrographs of the vacuoles revealed that they were membrane-bound, frequently multivesicular, and had a clear lumen.[4] At 4 weeks of age, single large intranuclear aggregates containing mutant ataxin-1 were detected in a subset of Purkinje cells.[5] The percentage of Purkinje cells that contained a large ataxin-1 aggregate increased throughout time, such that by 12 weeks of age 90% of the cells contained an aggregate.[5] Also by 4 weeks of age many of the Purkinje cells had eccentric nucleoli.[5] By 5 weeks of age, a loss of proximal dendrites and shrinkage of the molecular layer became evident (P.J. Skinner, University of Minnesota, unpublished data).[4] By 6 weeks of age, the nuclei of many Purkinje cells were severely invaginated,[6] and by 8 weeks of age, mild gliosis was detected in the molecular layer.[4] After the onset of ataxia, which is first detectable at 12 weeks of age, heterotopic Purkinje cells become detectable in the molecular layer of the cerebellum.[4,6] At 24 weeks of age, Purkinje cell loss became evident.[4] Thus, significant neuropathology develops in the Purkinje cells of *SCA1* transgenic mice before the onset of ataxia.

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Furthermore, the onset of ataxia occurs before there is detectable loss of Purkinje cells.

Transgenic mice expressing a variant form of mutant ataxin-1 with a nonfunctional nuclear localization signal revealed that mutant ataxin-1 has to enter the nucleus of a Purkinje cell to cause disease.[6] In another series of *SCA1* transgenic mice, a form of mutant ataxin-1 lacking a portion of its self-association region was expressed in Purkinje cells. These mice developed disease in the absence of detectable nuclear aggregates despite nuclear expression of ataxin-1.[6] Thus, although the localization of mutant ataxin-1 to the nucleus is required for disease, the formation of nuclear aggregates of ataxin-1 is not.

Recently, Lin and colleagues[7] used a PCR-based subtractive cDNA cloning approach and demonstrated that mutant ataxin-1, very early in the disease process, induces alterations in gene expression in both *SCA1* transgenic mice and SCA1 patients. This altered expression of genes likely contributes to the neuropathological alterations and eventual dysfunction of the Purkinje cells.

The coexistence of cytoplasmic vacuoles and dendritic atrophy in the Purkinje cells of *SCA1* transgenic mice raises the possibility that these two pathological features are in some way related. To investigate this hypothesis, we examined the subcellular distribution of somatodendritic membrane proteins in Purkinje cells of *SCA1* mice. The results clearly indicated that the cytoplasmic vacuoles contain proteins typically located in the somatodendritic membrane, supporting the idea that the vacuoles are derived from the somatodendritic membrane of Purkinje cells. Moreover, the localization of components of the ubiquitin-proteasomal pathway (UPP) to the vacuoles suggested that the vacuoles are a site of protein degradation.

Materials and Methods

Immunofluorescent Staining of Mouse Cerebella

Sections were generated and stained as described.[5] Mice were anesthetized and perfused with phosphate-buffered saline (PBS)-buffered formalin. The brains were immersed in PBS-buffered formalin and further fixed overnight, transferred to PBS, and stored at 4°C. Fifty-μm vibratome sections were cut from the midline sagittal plane of cerebella. Free-floating sections were blocked overnight with 2% normal goat serum in PBS with 0.3% Triton X-100 and then incubated with primary antibodies in blocking solution for 2 days on a rocker at 4°C. Sections were washed with PBS four times, for at least 20 minutes, and incubated in secondary antibodies in blocking solution for 2 days on a rocker at 4°C. Sections were washed with PBS four times for at least 20 minutes, and mounted on slides using glycerol-gelatin (Sigma Chemical Co., St. Louis, MO) containing 4 mg/ml n-propylgalate. Antibodies used were: a monoclonal anti-calbindin CL300 (1:500; Sigma) and a polyclonal anti-calbindin D-28 antibody (1:500; Chemicon, Temecula, CA), a rabbit anti-protein kinase C (PKC)γ antibody (1:1000, Sigma), a rabbit anti-20S proteasome core antibody (1:1000, Afiniti), and an anti-ubiquitin (DAKO, Carpinteria, CA). Goat anti-rabbit and anti-mouse antibodies conjugated to either Cy2 or Cy3 (1:500; Jackson ImmunoResearch, West Grove, PA), or goat anti-mouse antibodies conjugated to Alexa 488 (1:500; Molecular Probes, Eugene, OR) were used as secondary antibodies. Sections were analyzed using a BioRad MRC 1000 confocal microscope (Bio-Rad, Richmond, CA). Images were processed using Confocal Assistant and Adobe Photoshop.

Immunohistochemical Staining of Mouse Cerebellum

Immunohistochemistry was performed on 40-mm cryostat or vibratome sections from formalin-perfused brains that were soaked overnight in 20% buffered sucrose before sectioning. For mGluR1, staining was performed using the ABC Elite kit (Vector Laboratories, Burlingame, CA): sections were blocked for 1 hour in normal serum, incubated overnight at room temperature (mGlur1, 1:500), washed four times for 5 minutes each in PBS, and incubated for 2 hours at room temperature with biotinylated anti-mouse antibodies (1:200). These were washed four times for 5 minutes in PBS, incubated for 1 hour with avidin-biotin-peroxidase complex reagent, washed 2 times for 5 minutes in PBS, and two times for 5 minutes each in Tris, pH 7.6, exposed for several minutes to diaminobenzidine substrate, washed four times for 5 minutes each in PBS, dehydrated, cleared, and mounted.

RNA and Protein Analysis

For Northern blot analysis, cerebellar RNA was isolated by the acid guanidinium thiocyanate-phenol/chloroform method,[8] electrophoresed in the presence of glyoxal, blotted, and probed with radiolabeled cDNAs. For Western blot analysis, cerebella were homogenized in lysis buffer (10% glycerol, 5% mercaptoethanol, 2.3% sodium dodecyl sulfate, 0.06 mol/L Tris, pH 6.8). A portion of the crude lysate was heated with loading buffer and electrophoresed, blotted, and probed with rabbit anti- PKCγ (1:1000) or preimmune serum (1:1000), all with 0.1% Tween-20. After incubating with anti-rabbit or anti-mouse horseradish peroxidase conjugate, bands were visualized by chemiluminescence (NEN Renaissance kit; Dupont-New England Nuclear, Boston, MA).

Results

The Cytoplasmic Vacuoles in SCA1 Transgenic Purkinje Cells Contain Proteins from the Dendritic Membrane

Electron microscopic analysis revealed that the cytoplasmic vacuoles were distended membranous structures.[9] Confocal microscopic analyses of the cytoplasmic vacuoles in *SCA1* transgenic Purkinje cells revealed that in some Purkinje cells, the vacuoles seemed to be contiguous with the outer membrane (Figure 1). This observa-

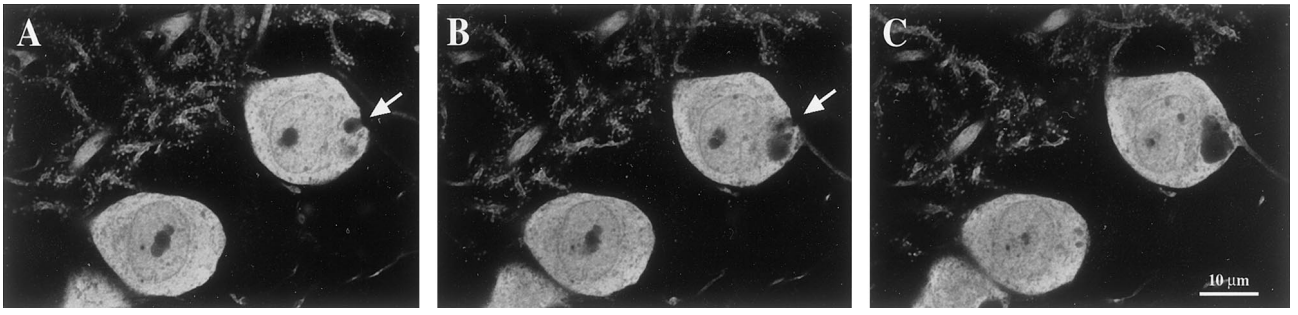

Figure 1. Cytoplasmic vacuoles in *SCA1* transgenic Purkinje cells can be visualized as congruent with the outer cell membrane. Images of three confocal Z scans collected at 1-μm intervals from a *SCA1* Purkinje cell counterstained with an antibody to calbindin. The **arrows** in **A** and **B** indicate the region where the cytoplasmic vacuole appears to be contiguous with the plasma membrane.

tion indicated that the cytoplasmic vacuoles are formed by the internalization of a substantial portion of the plasma membrane.

To further assess whether the formation of the cytoplasmic vacuoles in the *SCA1* mouse Purkinje cells involved the internalization of the outer cellular membrane, as well as to determine whether the formation of vacuoles was related to the concurrent loss of the dendritic tree, we undertook a series of immunofluorescence studies using markers for several Purkinje cell membrane proteins. We first examined whether the vacuoles were reactive to wheat germ agglutinin and, thus, contain complex carbohydrate-linked proteins from either the Golgi apparatus or cellular membrane. Figure 2A shows that the vacuole in a *SCA1* Purkinje cell stained strongly with wheat germ agglutinin (WGA), as well as the outer membranes of many cells in the cerebellar cortex. The WGA was typically found associated with membrane of the vacuoles as indicated in Figure 2A, but was also occasionally found as a bright focus within the lumen of vacuoles. The vacuoles were found not to be reactive to concanavalin A, an endoplasmic reticulum marker, and were not stained by antibodies to cathepsin D, a lysosomal marker, antibodies to the p58 Golgi protein, or markers to multivesicular vesicles (data not presented). From these data, it was concluded that the vacuoles were likely derived from the somatodendritic membrane.

A protein that is prominently expressed within the Purkinje cell dendritic membrane is the glutamate receptor mGluR1.[10] If the vacuoles are derived from the somatodendritic membrane compartment, it would be expected that they contain mGluR1. Figure 2B shows that a vacuole present in a 9-week-old B05/+ *SCA1* Purkinje cell was immunoreactive for mGluR1. Within the vacuoles, the mGluR1 staining was present in multiple small, typically less than 0.5-μm aggregates that primarily associated with the vacuolar membrane. In addition, the vacuoles were found to be immunoreactive for the dendritic membrane proteins GluRΔ1/Δ2 (Figure 2C) and for GluR2/3 (Figure 2D). The staining of GluRΔ1/Δ2 (Figure 2C), and GluR2/3 was typically found in 1 μm in diameter or smaller aggregates and these aggregates were found both associated with the vacuole membrane as well as within the lumen of the vacuoles. Thus, by immunolocalization analyses the cytoplasmic vacuoles in B05/+ Purkinje cells contained several proteins and markers typically found in the somatodendritic membrane compartment. Therefore, the cytoplasmic vacuoles are very likely derived from the somatodendritic membrane.

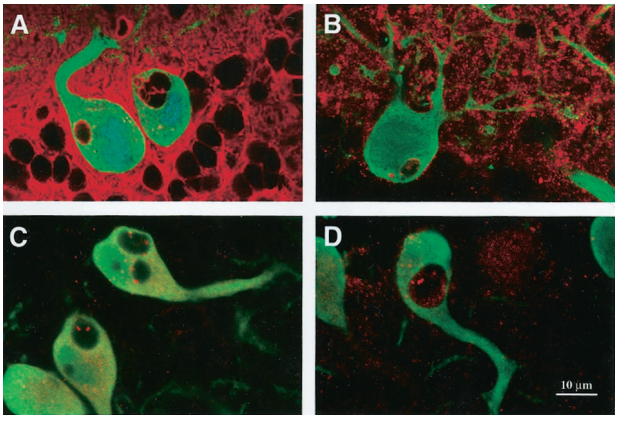

Figure 2. The cytoplasmic vacuoles in Purkinje cells of *SCA1* transgenic mice contain somatodendritic membrane proteins. Immunofluorescent analyses (red) of cerebellar sections from 10-week-old *SCA1* B05/+ mice demonstrating that the vacuoles are positive for WGA (**A**), mGluR1 (**B**), GluR Δ1/Δ2 (**C**), and GluR2/3 (**D**). Green corresponds to immunoreactivity to the Purkinje cell-specific protein calbindin.

Distribution of Membrane-Associated PKCγ in Transgenic SCA1 Purkinje Cells

PKCγ is expressed highly within Purkinje cells in the cerebellar cortex.[11] Because the translocation of PKCγ to the cell membrane is associated with its activation,[12–14] we were interested in determining the subcellular distribution of the PKCγ in *SCA1* Purkinje cells, particularly its membrane-associated form. Sections from 10-week-old wild-type and B05/+ *SCA1* animals were examined by confocal microscopy after immunofluorescence staining of the Purkinje cell-specific protein calbindin and PKCγ. Figure 3A shows the robust dendritic tree of a 10-week-old wild-type Purkinje cell. The substantial atrophy of the dendritic tree is revealed by calbindin immunoreactivity in a B05/+ 10-week-old Purkinje cell (Figure 3B). In a wild-type Purkinje cell, PKCγ was localized to the cytoplasm of the Purkinje cell body and to the membrane of the primary and secondary dendrites throughout the ex-

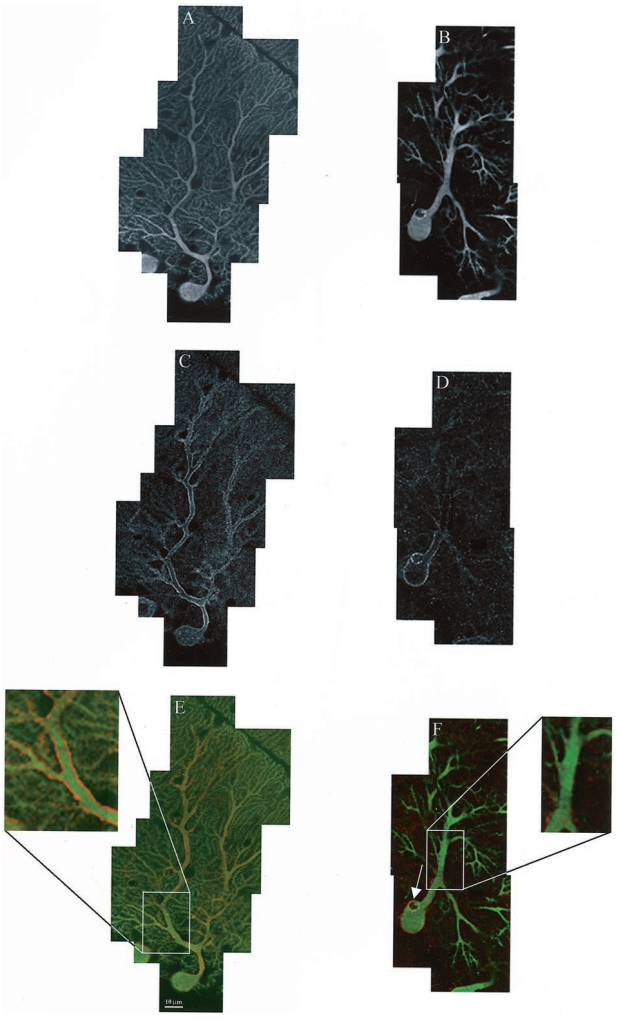

Figure 3. The subcellular distribution of PKCγ is altered in Purkinje cells of *SCA1* transgenic mice. By immunofluorescence, the Purkinje cell-specific protein calbindin is distributed throughout the Purkinje cells, including the dendritic tree, in a 10-week-old wild-type (**A**) and in a *SCA1* B05/+ mouse (**B**). **C** and **D:** The subcellular distribution of PKCγ in the 10-week-old wild-type and *SCA1* B05/+, respectively. **C:** PKCγ is localized to the cytoplasm throughout the Purkinje cell. In addition, some PKCγ is localized to the inner surface of the plasma membrane primarily in the primary and secondary dendrites. In contrast, in a aged-matched Purkinje cell from the *SCA1* mouse PKCγ expression is essentially undetectable in the dendrites and in the cell body PKCγ is localized to the plasma membrane. The merged images (calbindin in green and PKCγ in red) are presented in the wild-type mouse (**E**) and *SCA1* mouse (**F**). The associated **insets** show the membrane localization of PKCγ in the wild-type Purkinje cell dendrite and its absence from the dendrite in the *SCA1* Purkinje cell. The **arrow** in **F** highlights a cytoplasmic vacuole that is immunoreactive for PKCγ.

tent of the dendritic tree (Figure 3C). In contrast to the distribution of PKCγ in wild-type murine Purkinje cells, by 10 weeks of age in B05/+ Purkinje cells, PKCγ expression was limited to the somatic membrane and did not extend into the dendritic tree (Figure 3; D–F). In addition, the cytoplasmic vacuoles were strongly immunoreactive for PKCγ. Within the vacuoles, PKCγ was typically associated with the membrane as indicated in Figure 3F. However, in some vacuoles, PKCγ was aggregated in the lumen of the vacuole and not associated with the membrane (data not shown).

To determine the time course of the alteration in PKCγ expression with disease progression in B05/+ *SCA1* Purkinje cells, cerebellar sections were examined by immunofluorescence at various ages (Figure 4). In wild-type Purkinje cells at 3 weeks of age (Figure 4A), at 10 weeks of age (Figure 4C), and at 13 weeks of age (Figure 4E) PKCγ was localized to the dendritic membranes. However, as early as 3 weeks of age, well before the development of ataxia,[4] B05/+ Purkinje cells had a discernible loss of membrane-associated PKCγ expression in the dendrites (Figure 4B). Thus, early in the disease process of *SCA1* mice, before the onset of ataxia, PKCγ normally found associated with the dendritic membrane was found associated with the somatodendritic membrane and internalized in cytoplasmic vacuoles.

The Cytoplasmic Vacuoles in SCA1 Transgenic Purkinje Cells Contain Components of the UPP

Because the cytoplasmic vacuoles of B05/+ *SCA1* Purkinje cells contained proteins typically found in the somatodendritic membrane, we reasoned that their formation might be related to the loss of the dendritic tree and, thus, might contain components of a protein-degradative pathway. One protein-degradative pathway that has a role in the pathological response to the expression of an expanded *SCA1* allele is the UPP.[15] At 5 weeks of age, all of the cytoplasmic vacuoles in B05/+ Purkinje cells were intensely immunoreactive for ubiquitin (Figure 5A). The ubiquitin immunoreactivity was found in large, 0.5 to 4 μm in diameter aggregates within the vacuoles. By 10 weeks, only 50% of the vacuoles were immunoreactive for ubiquitin and the staining was less intense (Figure 5B). Perhaps reflecting the degradation of the ubiquitinated proteins in the vacuoles. Therefore, we next ascertained whether the proteasome apparatus also localized to the ubiquitin-positive vacuoles. Figure 5, C and D, shows that B05/+ cytoplasmic vacuoles were immunoreactive for the anti-20S proteasome. The proteasome staining showed multiple small, less that 0.5 μm in diameter, aggregates around the periphery of each vacuole. The size and abundance of the proteasome aggregates was increased in sections from a 10-week-old animal (Figure 5D) compared to sections from a 5-week-old animal (Figure 5C). Thus, the proteins located within the cytoplasmic vacuoles may be undergoing ubiquitin-mediated degradation.

Loss of PKCγ Was Primarily because of Protein Degradation

To gain insight into a molecular basis for the loss of PKCγ in *SCA1* transgenic cerebella, Western and Northern blot analyses were performed. Figure 6A shows that in B05/+ mice at 3 weeks of age, relative to wild-type cerebellum, there was a slight decrease in the amount of PKCγ protein detectable by Western blot analysis. Thus, early on the primary change seen in PKCγ expression was a redistribution from the dendritic membrane to the Purkinje cell body (Figure 4B). However, as the *SCA1* mice aged

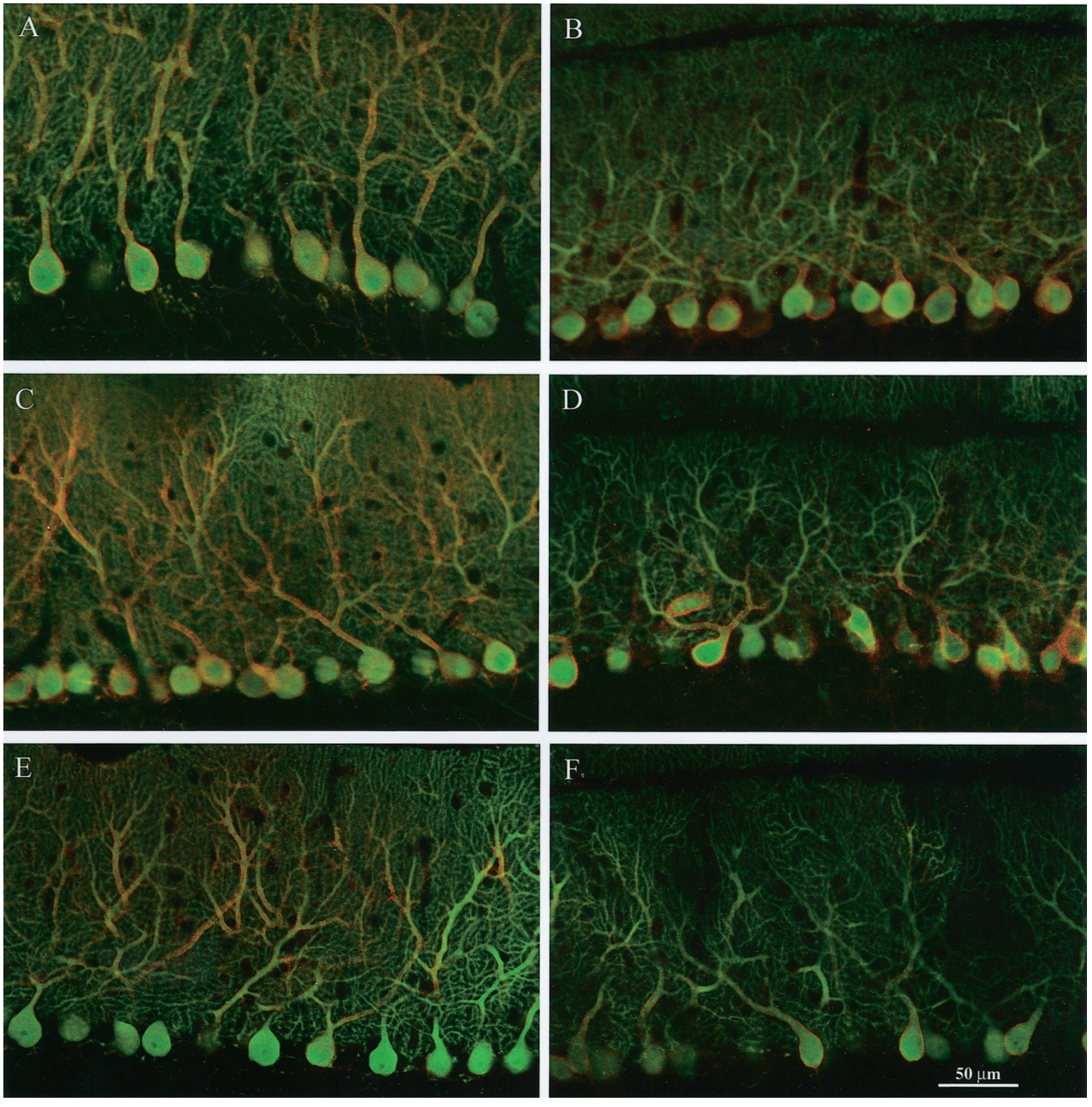

Figure 4. The loss of dendrite-associated PKCγ occurs before the onset of ataxia in *SCA1* transgenic mice. Immunoreactivity to the calbindin (green) and PKCγ (red) is shown in cerebellar sections from a 5-week-old wild-type mouse (**A**), a 5-week-old *SCA1* B05/+ mouse (**B**), a 10-week-old wild-type mouse (**C**), a 10-week-old *SCA1* B05/+ mouse (**D**), a 13-week-old wild-type mouse (**E**), and a 13-week-old *SCA1* B05/+ mouse (**F**). Loss of dendritic PKCγ was detected by 5 weeks of age in the *SCA1* cerebellum.

there was a detectable loss in absolute levels of PKCγ mRNA and more notably PKCγ protein. By 13 weeks of age the level of PKCγ protein was barely detectable (Figure 6A). Importantly, although Northern blot analyses at 3 and 12 weeks of age (Figure 6C) showed a slight decrease in PKCγ mRNA, the magnitude of this change is insufficient to explain the profound decrease in PKCγ protein shown by Western blotting. Thus, the loss in PKCγ mRNA was considerably less than the changes seen at the protein level. These data indicate that a substantial proportion of the loss of PKCγ protein in B05/+ Purkinje cells is the result of a posttranscription mechanism, such as an increase in protein turnover.

mGlur1 Is Maintained in the Dendrites of B05/+ SCA1 Purkinje Cells

Because we found that PKCγ protein levels decreased dramatically in the *SCA1* mouse Purkinje cells, we thought it would be important to determine the specificity of this change. As described above, the G-protein-cou-

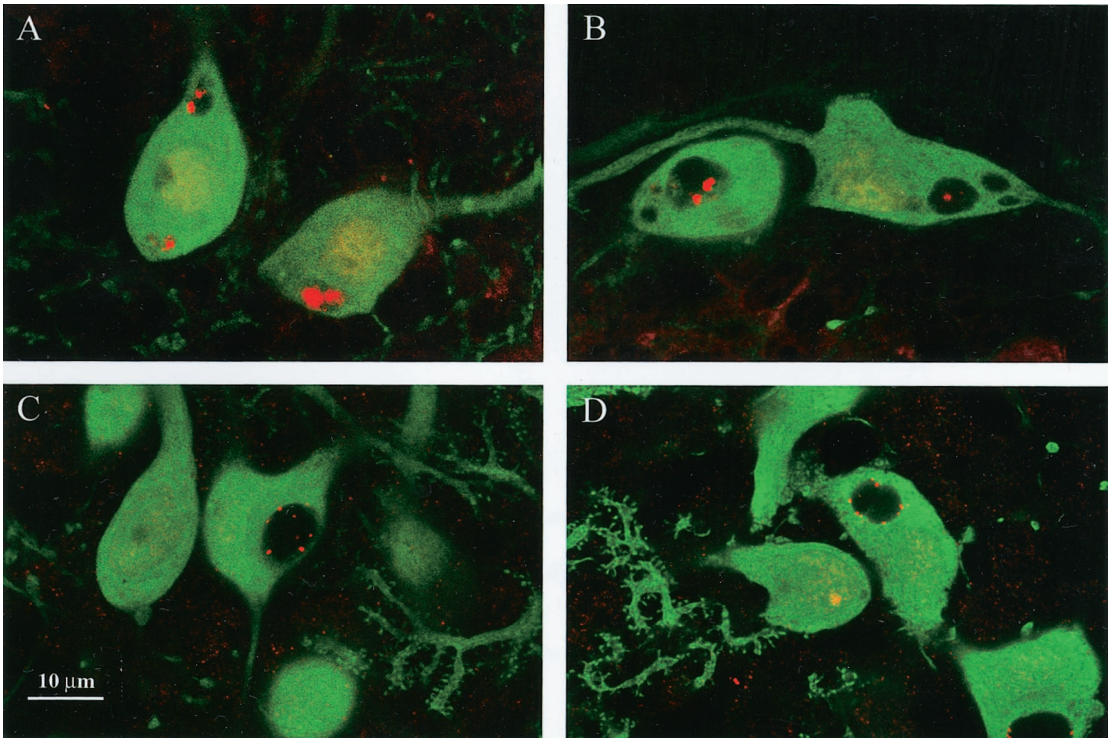

Figure 5. Components of the UPP localized to the cytoplasmic vacuoles in Purkinje cells of *SCA1* transgenic mice. **A** and **B:** The immunoreactivity (red) of the cytoplasmic vacuoles in *SCA1* B05/+ Purkinje cells for the 20s proteasomal component at 5 and 10 weeks of age, respectively. **C** and **D:** The immunoreactivity of cytoplasmic vacuoles in *SCA1* B05/+ Purkinje cells for ubiquitin at 5 and 10 weeks of age, respectively. Green corresponds to immunoreactivity to the Purkinje cell-specific protein calbindin.

pled glutamate receptor mGluR1 is abundant in Purkinje cell dendrites.[10] mGluR1 is a postsynaptic, G-protein-coupled receptor that activates phospholipase C,[16] thus playing a key role in the activation of PKCγ by diacylglycerol (DAG) in Purkinje cells. An absence of mGluR1 in mice results in motor impairment and disruption of cerebellar synaptic plasticity.[17,18]

To assess the status of mGluR1 in B05/+ Purkinje cells, an immunohistochemical analysis was performed. By this analysis, mGluR1 was readily detectable in Purkinje cell dendrites of 10-week-old B05/+ mice (Figure 7B), very similar to that seen in 10-week-old wild-type Purkinje cells (Figure 7A). Although the thickness of the molecular layer was considerably less in the B05/+ *SCA1* cerebellum because of the atrophy of the Purkinje cell dendrites at this age,[4] mGluR1 was detectable throughout the extent of B05/+ Purkinje cell dendrites. Thus, although the vacuoles were immunoreactive for mGluR1, the immunohistochemical analysis indicated that mGluR1 was not progressively lost from the Purkinje cell dendrites as was seen with PKCγ. It is also worth noting that the cytoplasmic Purkinje cell-specific protein, calbindin, by immunofluorescence (Figure 5) was retained in *SCA1* mice well beyond the age at which PKCγ became undetectable.

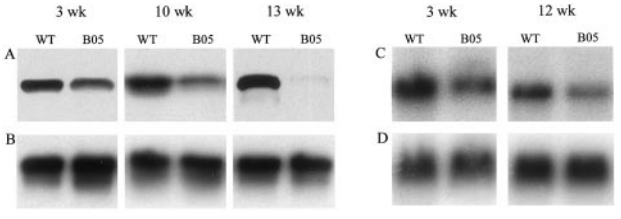

Figure 6. Posttranslational loss of PKCγ in *SCA1* transgenic mice. Western blot analyses of PKCγ in cerebellar protein extracts from wild-type (WT) and *SCA1* B05/+ mice at 3, 10, and 13 weeks are shown for PKCγ in **A** and for neurofilament as a loading control in **B**. Northern blots of cerebellar RNA from WT and *SCA1* B05/+ mice at 3 and 12 weeks are shown for PKCγ in **C** and for GAPDH as a loading control in **D**. These results demonstrate that cerebellar PKCγ protein levels decreased more rapidly than PKCγ RNA in *SCA1* transgenic mice.

Discussion

Two prominent pathological alterations that occur early in the disease process in *SCA1* transgenic mice are the formation of cytoplasmic vacuoles and the progressive loss of dendritic arborization in cerebellar Purkinje cells.[4] Our present studies indicate that these pathologies may be linked. First, we observed that in some Purkinje cells, the vacuoles were congruent with the outer cell membrane and invaginated into the cytoplasm (Figure 1). Second, we found that the vacuoles were stained with a marker of the outer cell membrane, wheat germ agglutinin, but not with markers specific to the Golgi apparatus, endoplasmic reticulum, or lysosomes. These data indicate that the vacuoles in the Purkinje cells of *SCA1* transgenic mice originate from the plasma membrane.

These observations led to the conclusion that the formation of vacuoles in the Purkinje cells of *SCA1* transgenic mice involves the internalization of the somatodendritic membrane. Moreover, these observations led to the

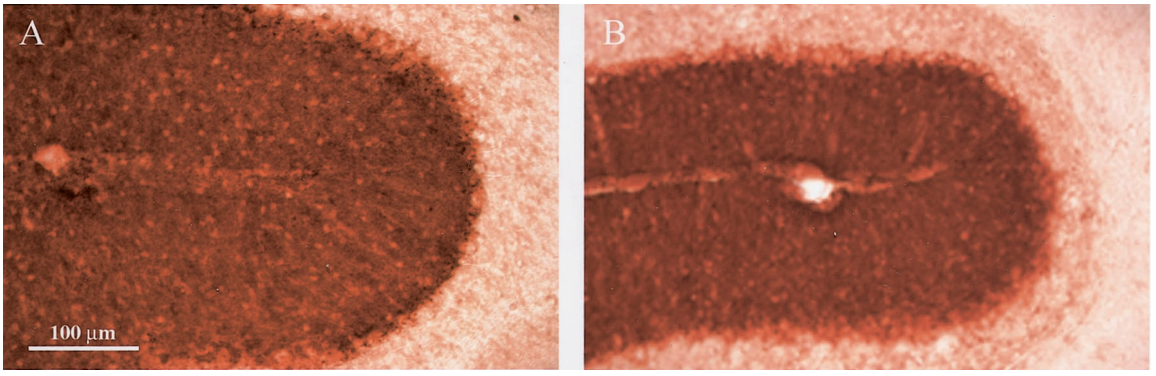

Figure 7. mGluR1 in *SCA1* transgenic mice is maintained in Purkinje cell dendrites. Immunohistochemical analyses of mGluR1 expression in the molecular layer, Purkinje cell dendrites in a 10-week-old wild-type (**A**) and a *SCA1* B05/+ (**B**) mouse cerebella.

hypothesis that the formation of vacuoles is the mechanism underlying the loss of dendritic membrane. In support of this hypothesis are the findings that 1) the time course of vacuole formation coincides with the loss of dendritic membranes; 2) the dendritic proteins mGluR1, GluRΔ1/Δ2, GluR2/3, and PKCγ, were detected in the cytoplasmic vacuoles; and 3) the vacuoles contained proteins of the UPP suggesting that the vacuoles are involved with the degradation of proteins. These points suggest that by a mechanism yet to be determined, mutant ataxin-1 induces the bulk internalization of Purkinje cell plasma membrane into vacuoles, which then recruit components of the UPP. As the internalization of plasma membrane proceeds, proteins associated with the dendrites are incorporated into the vacuoles and degraded, leading to the dendritic atrophy characteristic of SCA1 pathology.

The role of the UPP in the degradation of cytosolic and nuclear proteins is well established.[19,20] The UPP also has an important role in the degradation of misfolded newly synthesized proteins that have been removed from the endoplasmic reticulum.[21] In both of these instances the UPP system acts on nonmembrane-associated proteins. There are also a growing number of examples in which the ubiquitin/proteasome system is involved in the endocytosis of proteins from the plasma membrane.[22–25] The present findings extend the role of the UPP to the degradation of proteins in vacuoles as part of the neurodegeneration induced by a disease-causing polyglutamine protein.

Several aspects of the membrane internalization are worth noting. In *SCA1* Purkinje cells, there is no evidence that specific membrane proteins are selected for internalization as seen in clathrin-dependent endocytosis. Rather, all of the membrane proteins that were assessed were found in the cytoplasmic vacuoles, suggesting that the membrane proteins are internalized by the bulk internalization of the plasma membrane from the Purkinje cell soma (Figure 1). Furthermore, clathrin-dependent endocytosis occurs at clathrin-coated pits giving rise to small coated vesicles of less than 150 nm. The large size of the vacuoles in the Purkinje cells of *SCA1* transgenic mice is more typical of a clathrin-independent endocytosis mechanism, such as macropinocytosis, that can lead to large endocytic vesicles as large as 5.0 mm in diameter.[27,28] These points argue that the vacuoles found in the *SCA1* transgenic Purkinje cells are not formed by clathrin-mediated endocytosis, and may be formed by a non-clathrin-mediated endocytosis mechanism.

By 13 weeks of age the levels of PKCγ were essentially undetectable by Western blot and immunofluorescence. In contrast, at 13 weeks of age mGluR1 and other components of the somatodendritic membrane were still readily detectable by immunohistochemistry. This analysis indicates that the loss of dendritic membrane proteins, eg, mGluR1, is protracted and likely follows the loss of dendritic arborization that is not complete until 27 weeks of age in the *SCA1* mice.[4] So why is PKCγ lost at a faster rate than the dendritic membrane proteins? Perhaps the relative enhanced rate of PKCγ degradation is correlated with its subcellular distribution in *SCA1* transgenic Purkinje cells. Early on, PKCγ membrane association shifts from being primarily dendritic to being at the plasma cell membrane (Figure 4). As discussed above, formation of the cytoplasmic vacuoles seems to be the result of the bulk internalization of membrane from the Purkinje cell soma. Thus, the redistribution of membrane-associated PKCγ to the Purkinje cell plasma membrane would enhance its uptake into the cytoplasmic vacuoles and potentiate its degradation by the UPP. Consistent with this suggestion was the fact that the vacuoles stained prominently for PKCγ (Figure 3F), much more than for mGluR1 (Figure 2B), whose localization was maintained at the dendritic membrane (Figure 7).

The changes in PKCγ subcellular distribution detected at 3 weeks postnatally in the *SCA1* mice are an early sign of an alteration in Purkinje cell function induced by the expression of a polyglutamine protein. In Purkinje cells of wild-type mice, a substantial amount of the dendritic PKCγ localized to the inner surface of the cell membrane. In the dendritic tree of transgenic *SCA1* Purkinje cells there was an almost complete loss of PKCγ associated with the cell membrane. Because the translocation of PKCγ to the membrane is a marker of its activation,[12–14] these data indicate that PKCγ normally is more active in the dendrites of Purkinje cells than in the cell body, and that an early event in the Purkinje cell disease of the *SCA1* mice is an alteration in the PKCγ dendritic-signaling pathway. PKC isoforms are activated sequentially by calcium and DAG.[12,26] Repetitive calcium spikes result in PKC

translocation to the membrane where binding to DAG seems to function in the retention of activated PKC at the plasma membrane. Thus, the observed absence of membrane-associated PKCγ in the dendrites of *SCA1* transgenic Purkinje cells could be because of a reduction in calcium spikes and/or reduction of DAG at the plasma membrane. A potential source of external calcium are voltage-gated calcium channels in the plasma membrane at the climbing fiber synapse,[27] and internally the inositol triphosphate-mediated release of calcium in the endoplasmic reticulum.[28–30] Both IP3 and DAG would be produced as a result of phospholipase C activation because of ligand interaction with a G-coupled receptor,[31] ie, parallel fiber release of glutamate that acts on metabotropic G-coupled receptor mGluR1 on Purkinje cells.[32] It has been demonstrated that these signals are followed by the activation of PKC, and that the activation of PKC is required for the induction of cerebellar long-term depression.[33]

It remains unclear the extent to which vacuole formation characterized in the *SCA1* transgenic mice correlates with disease process in SCA1 patients. Purkinje cell cytoplasmic vacuoles have not been reported in SCA1 patient material.[34] However, SCA1 patient pathological studies have been limited to autopsy material obtained at the end stage of disease, long after most Purkinje cells have disappeared. In aged *SCA1* mice, when the Purkinje cell dendrites were essentially gone, the cytoplasmic vacuoles were no longer detected. Whether cytoplasmic vacuoles are a component of the disease in humans awaits examination of postmortem tissue form SCA1 patients at an early stage of disease, before the complete loss of the Purkinje cell dendritic tree.

Although the results reported here provide important insights into the molecular functions whose alteration is associated with the cytoplasmic pathology seen in Purkinje cells of *SCA1* transgenic mice, they need to be placed in a broader picture of SCA1 pathogenesis in which the nucleus is clearly the site of the initial disease-causing events.[6] Thus, the cytoplasmic alterations seen in the transgenic *SCA1* Purkinje cells are likely triggered by mutant ataxin-1-induced events in the nucleus. The most likely scenario is that the cytoplasmic alterations are in response to changes in gene expression induced by mutant ataxin-1.[7] The number and identity of those genes whose altered expression results in the altered trafficking of somatodendritic membrane proteins seen in *SCA1* Purkinje cells remain to be determined.

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